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Current hypotheses on the mechanisms of toxicity of ultrafine particles

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Summary. - PM_{10} is a complex mixture of particles and we have focused here on the ultrafine component, i.e. particles with a diameter of less than 100 nm. In PM_{10} this fraction is mostly composed of combustion-derived, carbon-centred particles with associated hydrocarbons and metals. Progress in understanding the effects of ultrafine particles in the lungs has been achieved largely through the use of surrogate particles such as ultrafine carbon black and titanium dioxide. Using these types of particles, ultrafines have been shown to cause oxidative stress and pro-inflammatory effects in a number of *in vivo* and *in vitro* models. The mechanisms of the generation of the oxidative stress is not understood, but appears to be related to the large particle surface area in some way. Modulation of calcium signalling also appears to be involved in the stimulation of cytokine release by macrophages in response to ultrafines. Effects of PM_{10} are seen on cardiovascular mortality and morbidity, as well as on the lung. Although the role of ultrafine particles in these effects are not well understood there are plausible pathways that remain to be explored.

Key words: PM_{10}, ultrafine particles, inflammation, calcium, oxidative stress.

Riassunto *(Ipotesi correnti sui meccanismi di tossicità delle particelle ultrafini)*. - L'oggetto del presente lavoro è la componente ultrafine, cioè la porzione con diametri inferiori a 100 nm, della complessa miscela di particelle costituente il PM_{10}. Nel PM_{10} questa frazione è essenzialmente composta di particelle originate da processi combustivi, con un nucleo centrale carbonioso a cui sono associati vari idrocarburi e metalli. I progressi nella comprensione degli effetti sui polmoni delle particelle ultrafini sono stati raggiunti in larga parte con l'uso di particelle surrogate, quali quelle di carbonio e di biossido di titanio ultrafini. Grazie a questi due tipi di particelle, è stato mostrato che le ultrafini causano stress ossidativo ed effetti proinfiammatori in diversi modelli *in vivo* e *in vitro*. I meccanismi che portano alla generazione di stress ossidativo non sono ancora ben conosciuti, ma sembrano correlati in qualche modo con l'area superficiale molto estesa di queste particelle. Nella risposta alle ultrafini, inoltre, la modulazione del segnale del calcio sembra coinvolta nella stimolazione del rilascio di citochine da parte dei macrofagi. Gli effetti negativi del PM_{10} sono stati riscontrati a carico della mortalità e morbidità cardiovascolare, nonché di altre patologie polmonari. Benché il ruolo delle particelle ultrafini nella determinazione di questi effetti non sia ancora ben compreso, sono individuabili delle ipotesi plausibili sono ancora da esplorare.

Parole chiave: PM_{10}, particelle ultrafini, infiammazione, calcio, stress ossidativo.

The PM_{10} problem and the possible role of ultrafine particles

There has been increased application and use of ultrafines in industry with concomitant potential for occupational exposure [1], but the greatest concern regarding ultrafine particles has emanated from research on particulate air pollution primarily on PM_{10} and $PM_{2.5}$. Environmental particulate air pollution is measured by a global sampling convention called PM_{10} that measures mass of particles collected with a 50% efficiency for particles with an aerodynamic diameter of 10 μm; a wide range of particle sizes are collected from coarse 10 μm particles down to the ultrafine size range. This closely corresponds to the International Standards Organisation (ISO) Thoracic Convention, i.e., the mass fraction of inhaled particles that penetrates beyond the larynx to the airways. The respirable fraction, which penetrates to the unciliated regions of the lung, is approximated by an analogous $PM_{2.5}$ convention, which is now being considered in the USA as the standard measurement for reasons discussed below [2].

Attention has focused on the PM_{10} in cities because that is where most deaths occur, where pollution is routinely monitored and hence the associations are best

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seen. Typical urban PM_{10} is comprised of up to 50% by mass of combustion-derived, ultrafine carbon-centred particles with associated metals including transition metals. Other major components include ammonium salts of nitrogen, sulphur and chlorine plus geological dust and organic matter [3].

There has been extensive discussion as to which components of the PM_{10} particle mix might be responsible for its associated adverse health effects. A number of the components of PM_{10} have been hypothesised to drive the toxicological effects, one such component being the ultrafine fraction [4, 5]. The combustion derived ultrafine particles have come under suspicion because of the general perception that most of the other components are relatively harmless at the measured exposure levels, plus toxicological evidence that ultrafine particles and metals have potential toxicity. Another component which has also been hypothesised to drive inflammation is endotoxin, a component of some PM samples, especially those collected in rural locations where wind blown soil is a major component of PM_{10}. Research has shown adverse effects of environmental particles at the levels found in European and UK cities, but there is heterogeneity in magnitude of the health effects estimates in different cities [6]. This heterogeneity is probably explained by the fact that PM_{10} is a highly complex mixture of different components that varies, as might be anticipate, between sites depending on the local conditions including weather, season, etc., and, of course, the potential for exposure both indoors as well as outdoors. Indoor PM_{10} is affected by the special conditions pertaining to the indoor environment under consideration e.g. cigarette smoke, hay dust, cooking-derived particles. Local practice, tradition and behaviour could greatly influence the indoor environmental conditions and hence PM exposure profiles.

Ultrafine particles in the air and the lungs

Many toxicological studies over the last ten years have confirmed that particles in the ultrafine size range (<100 nm) pose special problems to the lungs (reviewed in [8-10]). Typically ultrafine particles cause more inflammation in experimental studies than larger respirable particles made from the same material when delivered at the same mass dose. Ultrafine particles are very small in relation to cellular structures and the average singlet particle of ultrafine is closer in scale to the ultrastructural/molecular size range than the cell scale [9]. Examples of aggregates of ultrafine particles collected from the atmosphere of UK cities are shown in Fig. 1.

Particle as small as ultrafine particles have a very large surface area and particle number per unit mass.

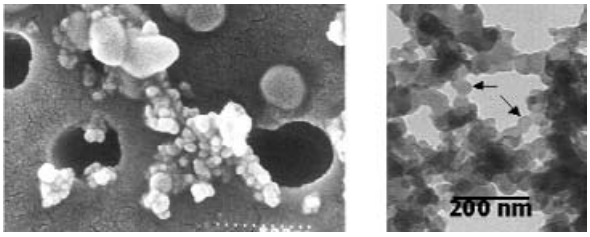

Fig. 1. - Aggregates of ultrafine particles in air from Birmingham (left, scanning electron microscope image, from the 3rd QUARG report) and Glasgow (right, transmission electron microscope image courtesy of Martin Wilson). In both cases there are large aggregates composed of singlet ultrafine (arrows) and larger particles.

To obtain 10 $\mu g/m^3$ of 2 μm diameter particles you only need 1.2 particles per ml of air and the total surface area of particles is 24 μm^2/ml; the same airborne mass concentration of 20 nm particles requires 2.4 million particles with a surface area of 3,016 μm^2/ml. The lung is likely to respond quite differently to 2.4 million particles with their concomitant huge surface area than to a relatively small number of larger particle. Ultrafine particles are present in large numbers in ambient air, with outside background levels in the range 5000-10,000 particles per ml rising during pollution episodes to 3,000,0000 particles/ml [5].

Since we spend more than 90% of our time indoors then the indoor environment should be considered and it is notable that vacuum cleaning has been reported to generate more than 8,000,000 particles/cubic foot [11]. Activities such as cooking also generate considerable numbers of ultrafine particles, especially in poorly ventilated environments [12].

Ultrafine particle deposit with high (50%) efficiency in the lungs [13]. Few studies have measured ultrafines in the environment and tried to relate them to adverse health effects, but in a group of asthmatics studied in Erfert, Germany, decrements in evening peak flow were associated with various size fractions of the airborne particles during a severe air pollution episode, but the best association was with the ultrafine fraction [14].

Oxidative stress and inflammation in the effects of PM_{10}

Inflammation is considered to play a major role in the adverse effects of PM_{10} such as exacerbations of airways disease [15] and also the cardiovascular effects [16]. Inflammation or pro-inflammatory effects have been described in:

- human subjects inhaling concentrated ambient particles [17];

- rats exposed to PM_{10} [18];
- cells exposed to PM_{10} *in vitro* [19].

Oxidative stress results from respiration and metabolism in biological systems and a number of antioxidant systems have evolved to provide protection against oxidative stress. Inflammation is a vital response which has evolved to deal with injury and to stimulate the regeneration of healthy tissue, although in an excessive amount or inappropriate setting inflammation is harmful leading to disease. In addition to being a consequence of normal cell respiration and metabolism, oxidative stress is a common accompaniment to cellular injury, e.g., injury induced by radiation, microbial proliferation, ischaemia, etc., and so evolution has built on this oxidative response to form a link between oxidative stress and inflammation. The response to the oxidative stress "danger signal" can be seen as having 3 separate parts:

1) a sensory arm that responds to the oxidative stress as indicated by increases in the production of molecules such as oxidised glutathione (GSSG) [20] and 4-hydroxynonenal [21], that are produced as a result of oxidation within the cell;

2) an effector arm that involves the increases in GSSG and 4-hydroxynonenal stimulating the activation of oxidative stress-responsive transcription factors such as NF-κB and AP-1 which then bind to DNA; formation of the transcription complex together with chromatin remodelling leads to transcription of key pro-inflammatory genes;

3) a response arm in the form of translated proteins such as the pro-inflammatory mediators, namely cytokines, and the proteins that act to remove the oxidative stress, namely antioxidants, e.g., superoxide dismutase (SOD), catalase or γ-glutamyl cysteinyl synthetase. Inflammation resulting from these events will also act to restore redox balance by removing the original source of the oxidative stress, but can also itself contribute to oxidative stress in a number of ways.

At the surface of particles a range of physicochemical reactions are thought to occur that result in intracellular oxidative stress. Due to the intimate contact encountered between deposited particles and pneumocytes there is direct delivery of this oxidative stress to cells on the lung surface. Furthermore, reactive oxygen species are generated during phagocytosis of the particles, leading to enhancement of oxidative stress.

The toxicology of ultrafine particles: the role of oxidative stress

The best evidence to support the "ultrafine hypothesis" for the adverse health effects of PM_{10} comes from toxicology. There is a substantial body of evidence to support the contention that ultrafine particles have extra toxicity and inflammogenicity compared to fine, respirable particles of the same material when delivered at the same mass dose. This has now been shown for a range of different materials of generally low toxicity, such as carbon black (CB) and titanium dioxide. Why a large number of particles or a large surface area leads to inflammation is not known, but surface area seems to be the metric that drives inflammation *in vivo* caused by low toxicity particles (Fig. 2) [22]. The particle surface may be a source of reactive oxygen species and this has been demonstrated *in vitro* for ultrafine and fine CB (Fig. 3) [23]. In this experiment fine or ultrafine particles were incubated with a compound that undergoes activation to a fluorescent state when it is oxidised. As shown in Fig. 3, ultrafine CB, but not CB, caused a dose dependent increase in fluorescence indicative of oxidation. We have demonstrated that this effect is not mediated by transition metals nor any other soluble agent [24], but is some consequence of the high surface area interacting with the biological system.

For some particles it is feasible that the inflammation could result from release of transition metals from the large surface area and in such situations transition metals like iron are considered to play an important role in causing inflammation *via* oxidative stress. Iron redox cycles between the I and II forms and in doing so generates hydroxyl radicals that cause severe oxidative stress. We have demonstrated that PM_{10} can generate hydroxyl radical via this mechanism [25]. Furthermore, we have found that ultrafine particles interact with iron salts to potentiate reactive species production in a cell free system, as well as to potentiate inflammation in the rat lung [23].

Ultrafine particles cause oxidative stress as demonstrated by a number of different assays and models:

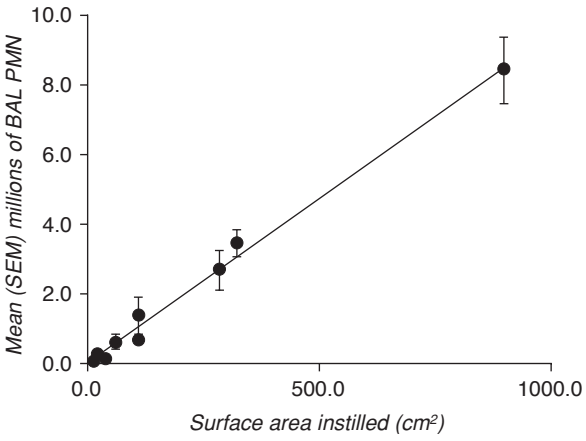

Fig. 2. - Inflammation caused by instillation of 125 or 1000 μg of low-toxicity particles, namely carbon black, titanium dioxide and polystyrene with the dose expressed as surface area.

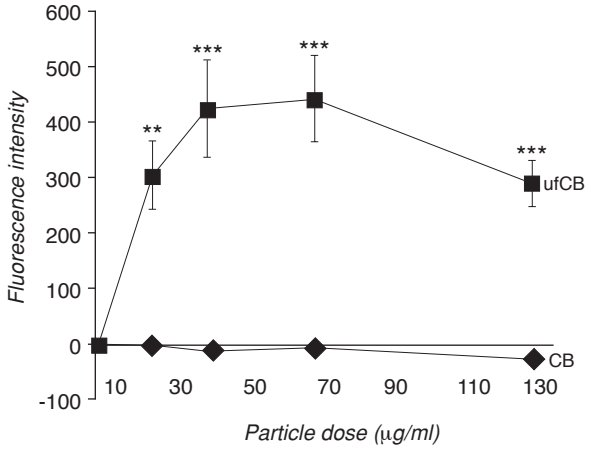

Fig. 3. - Results of an experiment to demonstrate the greater oxidising activity of ultrafine particles in a cell-free system. Modified from [23]. Asterisks denote significant differences between ultrafine carbon black (ufCB) and fine carbon black (CB).

- epithelial cells in culture were exposed to fine and/or ultrafine CB and depletion of GSH. A key antioxidant only occurred in the case of ufCB [26];

- rats instilled with ultrafine carbon black showed greater inflammation and depletion if GSH in the lung lavage fluid than was seen with fine CB [18];

- in a number of cell-free tests for the generation of free radicals, the ultrafine CB particles produce much more oxidation than CB particles [23, 27];

- instillation of ultrafine particles into the rat lung with an antioxidant nacystelin, reduced the inflammation by up to 60% compared to rats instilled with the particles alone [28].

Ultrafine particles of CB are also more potent at inhibiting phagocytosis by macrophage cells than larger respirable CB particles [29]. Decreased phagocytosis induced by the ultrafine component of a mixed dust would allow augmented interaction between the other particle components and the epithelium and a build-up of "dose" within the lung; both of these could enhance inflammation and hence would be important in PM_{10} exposure.

Hypothetical cellular events leading to pro-inflammatory gene transcription after ultrafine particles

Experiments are under way to elucidate the cellular and molecular events resulting from encounters between ultrafine particles and cells, but based on current knowledge the following sequence of events are hypothesised to occur (Fig. 4). Particles cause oxidative stress to the cells [26] with the generation of lipid peroxidation products such as 4 hydroxynonenal and the generation of GSSG, the oxidised form of GSH. This change in the redox balance of the cell towards oxidation can result in the acetylation of histones that loosens the contact between DNA and histone and allows access of the transcriptional complex to the promoter region to allow transcription to occur [30]. At the same time oxidative stress also causes the transcription factor NF-κB to translocate to the nucleus and gain access to the promoter region of key genes [19]. The genes that are controlled by NF-κB include TNFα, IL-8, IL-2, IL-6, GM-CSF, TNFα, ICAM-1, E-selectin and inducible nitric oxidase synthase (iNOS) and so activation of the NF-kB system can be seen to be highly pro-inflammatory [31]. Furthermore, either the oxidative stress and/or the direct particle interaction stimulates an increase in cytosolic Ca^{2+} concentration [32] that may also cause activation of NF-κB [33]. Calcium signalling events may also drive the further production of reactive oxygen species leading to a positive feedback mechanism [34]. A combination of these events may culminate in the transcription of genes that lead to inflammation as well as an increase in antioxidant production. For example, ultrafine CB treatment of both macrophages *in vitro* stimulated the production of TNFα protein production, a response which can be inhibited by several antagonists which block intracellular calcium signalling [35]. These events are summarised in Fig. 4.

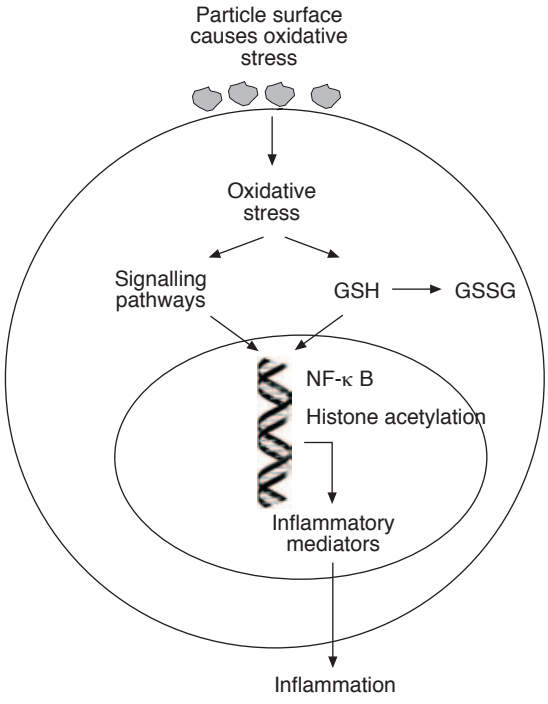

Fig. 4. - Diagram of the hypothetical events that lead to transcription of pro-inflammatory genes in particle-exposed cells.

The cardiovascular effects of particles

There is a clear intuitive relationship between the inflammatory effects of PM_{10} and the exacerbation of airways diseases such as COPD and asthma, but the relationship between particles in the lungs and cardiovascular effects is more difficult to understand. However, there are a number of plausible hypotheses to explain the effects on the cardiovascular system. These are summarised in Fig. 5. On the left side of the diagram are hypothetical effects that culminate in changes in heart rhythm as particles either stimulate the autonomic nervous system or cause direct or indirect damage to the heart. On the right are hypothetical pathways that lead from particles in the lung to ischaemic events involving enhanced clotting, haemostasis and atheromatous plaque rupture. The role of ultrafine particles, or indeed any of the individual components of PM_{10}, in these events is not clear. There is limited evidence for some of these pathways, but more research is needed before the actual events that lead from particles depositing in the lungs to cardiovascular deaths is clarified.

Conclusions

We can draw the following conclusions regarding the mechanisms of the adverse effects of ultrafine particles:

- there is good toxicological evidence that ultrafine particles cause inflammation in the lungs even when composed of relatively low toxicity materials.

- the mechanism of the induction of inflammation appears to be *via* oxidative stress and Ca^{2+} signalling perturbations;

- ultrafine particles can also inhibit phagocytosis more than the same mass of fine particles;

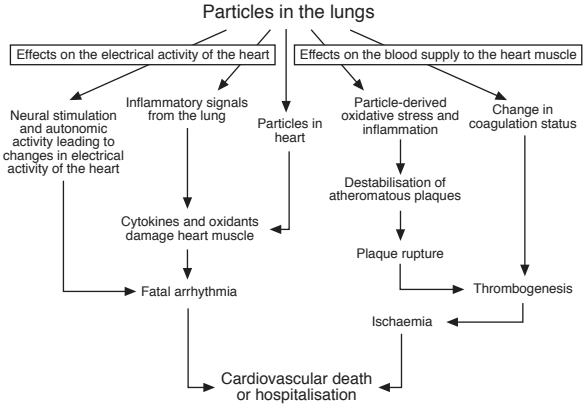

Fig. 5. - Hypothetical events that link particles exposure via the lungs to adverse cardiovascular effects.

- there are large numbers of ultrafine particles in environmental air (PM_{10}) mixed with a cocktail of other particulate components;

- increases in PM_{10} are associated with attacks of airways disease and deaths from cardiovascular and respiratory causes;

- the exact role that the ultrafine component of PM_{10} plays in the adverse effects of PM_{10} remains unknown.

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REFERENCES

1. Pui DH, Chen DR. Nanometer particles: A new frontier for multidisciplinary research. *J Aerosol Sci* 1997;28:481-760.

2. Donaldson K, Tran CL, MacNee W. Deposition and effects of fine and ultrafine particles in the respiratory tract. *Eur Resp Mon* 2002;7:77-92.

3. Harrison RM, Jones M. The chemical-composition of airborne particles in the uk atmosphere. *Sci Total Environ* 1995;168:195-214.

4. MacNee W, Donaldson K. Particulate air pollution:injurious and protective mechanisms in the lungs. In: Holgate ST, Samet JM, Koren HS, Maynard RL (Ed.). *Air pollution and health*. San Diego: Academic Press; 1999. p. 653-72.

5. Utell MJ, Frampton MW. Acute health effects of ambient air pollution: the ultrafine particle hypothesis. *J Aerosol Med* 2000; 13:355-9.

6. Katsouyanni K, Touloumi G, Samoli E, Gryparis A, Le Tertre A, Monopolis Y, Rossi G, Zmirou D, Ballester F, Boumghar A, Anderson HR, Wojtyniak B, Paldy A, Braunstein R, Pekkanen J, Schindler C, Schwartz J. Confounding and effect modification in the short-term effects of ambient particles on total mortality: results from 29. European cities within the APHEA2 project. *Epidemiology* 2001;12:521-31.

7. Pope CA. Epidemiology of fine particulate air pollution and human health: biologic mechanisms and who's at risk? *Environ Health Perspect* 2000;108(Suppl 4):713-23.

8. Donaldson K, Stone V, MacNee W. The toxicology of ultrafine particles. In: Maynard RL, Howards CV (Ed.). *Particulate matter: properties and effects upon health*. Oxford: Bios; 1999. p. 115-27.

9. Donaldson K, Stone V, Clouter A, Renwick L, MacNee W. Ultrafine particles. *Occup Environ Med* 2001;58:211-6.

10. Donaldson K, Stone V, Gilmour PS, Brown DM, MacNee W. Ultrafine particles: mechanisms of lung injury. *Phil Trans R Soc. London A* 2000;358:2741-829.

11. Lioy PJ, Wainman T, Zhang J, Goldsmith S. Typical household vacuum cleaners: the collection efficiency and emissions characteristics for fine particles. *J Air Waste Manag Assoc* 1999;49: 200-6.

12. Dennekamp M, Howarth S, Dick CA, Cherrie JW, Donaldson K, Seaton A. Ultrafine particles and nitrogen oxides generated by gas and electric cooking. *Occup Environ Med* 2001;58: 511-6.

13. Anderson PJ, Wilson DJ, Hirsch A. Respiratory tract deposition of ultrafine particles in subjects with obstructive or restrictive lung disease. *Chest* 1990;97:1115-20.

14. Peters A, Wichmann HE, Tuch T, Heinrich J, Heyder J. Respiratory effects are associated with the number of ultrafine particles. *Am J Resp Crit Care Med* 1997;155:1376-83.

15. MacNee W, Donaldson K. Particulate air pollution: injurious and protective mechanisms in the lungs. In: Holgate ST, Samet JM, Koren HS, Maynard RL (Ed.). *Air pollution and health*. San Diego: Academic Press; 1999. p. 653-72.

16. Donaldson K, Stone V, Seaton A, MacNee W. Ambient particle inhalation and the cardiovascular system: potential mechanisms. *Environ Health Perspect* 2001;109 (Suppl 4):523-7.

17. Ghio AJ, Kim C, Devlin RB. Concentrated ambient air particles induce mild pulmonary inflammation in healthy human volunteers. *Am J Resp Crit Care Med* 2000;162:981-8.

18. Li XY, Gilmour PS, Donaldson K, MacNee W. Free radical activity and pro-inflammatory effects of particulate air pollution (PM_{10}) *in vivo* and *in vitro*. *Thorax* 1996;51:1216-22.

19. Jimenez LA, Thompson J, Brown DA, Rahman I, Antonicelli F, Duffin R, Drost EM, Hay RT, Donaldson K, MacNee W. Activation of NF-kappaB by PM_{10} occurs *via* an iron-mediated mechanism in the absence of IkappaB degradation. *Toxicol Appl Pharmacol* 2000;166:101-10.

20. Rahman I, MacNee W. Regulation of redox glutathione levels and gene transcription in lung inflammation: therapeutic approaches. *Free Radic Biol Med* 2000;28:1405-20.

21. Esterbauer H, Schaur RJ, Zollner H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic Biol Med* 1991;11:81-128.

22. Duffin R, Clouter A, Brown DM, Tran CL, MacNee W, Stone V, Donaldson K. The importance of surface area and specific reactivity in the acute pulmonary inflammatory response to particles. *Ann Occup Hyg* 2002;46 (Suppl 1):242-5.

23. Wilson MR, Lightbody JH, Donaldson K, Sales J, Stone V. Interactions between ultrafine particles and transition metals *in vivo* and *in vitro*. *Toxicol Appl Pharmacol* 2002;184:172-9.

24. Brown DM, Stone V, Findlay P, MacNee W, Donaldson K. Increased inflammation and intracellular calcium caused by ultrafine carbon black is independent of transition metals or other soluble components *Occup Environ Med* 2000;57:685-91.

25. Gilmour PS, Brown DM, Lindsay TG, Beswick PH, MacNee W, Donaldson K. Adverse health-effects of PM_{10} Particles involvement of iron in generation of hydroxyl radical. *Occup Environ Med* 1996;53:817-22.

26. Stone V, Shaw J, Brown DM, MacNee W, Faux SP, Donaldson K. The role of oxidative stress in the prolonged inhibitory effect of ultrafine carbon black on epithelial cell function. *Toxicol In Vitro 1*998;12:649-59.

27. Wilson M, Lightbody JH, Donaldson K, Sales JH, Stone V. Interactions between ultrafine particles and transition metals *in vivo* and *in vitro*. *Toxicol Appl Pharmacol* 2002; submitted.

28. Donaldson K, Stone V, Gilmour PS, Brown DM, MacNee W. Ultrafine particles: mechanisms of lung injury. *Philos Transac Mathematical, Phys & Engin Sci* 2000;358:2741-9.

29. Renwick LC, Donaldson K, Clouter A. Impairment of alveolar macrophage phagocytosis by ultrafine particles. *Toxicol Appl Pharmacol* 2001;172:119-27.

30. Gilmour PS, Rahman I, Donaldson K, MacNee W. Histone acetylation regulates epithelial IL-8 release mediated by oxidative stress from environmental particles. *Am J Physiol Lung Cell Mol Physiol* 2003;284:L533-40.

31. Li N and Karin M. Is NF-kappaB the sensor of oxidative stress? *FASEB J* 1999;13:1137-43.

32. Stone V, Tuinman M, Vamvakopoulos JE, Shaw J, Brown D, Petterson S, Faux SP, Borm P, MacNee W, Michaelangeli F, Donaldson K. Increased calcium influx in a monocytic cell line on exposure to ultrafine carbon black. *Eur Respir J* 2000;15: 297-303.

33. Dolmetsch RE, Xu K, Lewis RS. Calcium oscillations increase the efficiency and specificity of gene expression *Nature* 1998;392:933-6.

34. Sen CK, Roy S, Packer L. Involvement of intracellular Ca^{2+} in oxidant-induced NF-kappa B activation. *FEBS Lett* 1996;385: 58-62.

35. Brown DM, Donaldson K, Stone V. Role of calcium in the induction of TNFα expression by macrophages on exposure to ultrafine uarticles. *Ann Occup Hyg* 2002;46(Suppl 1):219-22.